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14. ABSTRACT

The goal of this grant is to establish a new biological system for studying the progression of prostate cancer. We propose a technology we have previously developed to help define X-chromosome inactivation to increase our understanding of the molecular biology of prostrate cancer. Using a mouse model, our goal is to induce functional Loss of Heterozygosity (LOH) on a particular chromosome at various specified times during development or life span. Specifically, we plan to induce LOH only in mouse prostatic tissues. We are particularly interested in evaluating sites of allelic loss previously identified to be associated with prostate cancer (7q, 8p, 10p, 10q, 13q, 16q, 18q) in humans and understanding the effect of LOH on syntenic mouse chromosomes. We believe this approach has the potential to increase our knowledge of the acquisition and progression of prostate cancer. A systematic experimental approach for creating LOH can help define new prostate specific tumor suppressor genes. As human chromosome 8p is most frequently associated with prostate cancer (80% of primary and metastatic prostate cancers show this chromosomal variant, our mouse model system will initially focus on the development of those mouse chromosomes syntenic with human chromosome 8p. This technology would augment the positional cytogenetic approach to understanding the genetic complexity of prostate cancer.

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5)Introduction

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6) Body

Background

Activation or inactivation of a gene may lead to carcinogenesis. Activation of genes refers to a dominant condition that results in stimulation of both growth and progression of cancer. Inactivation of genes refers to the phenomena where tumor supressor genes (genes that normally inhibit carcinogenesis) are inactivated resulting in a loss function. Classically, these mutations are due to lesions which alter the linear sequence of a particular gene. In addition, somatic dysregulation or inappropriate gene-silencing due to methylation (where the gene is present but nonfunctional), may have similar effects.

Inactivation of a gene may occur as a result of allelic deletion where one or both copies of a locus is lost (1-4). Most frequently one copy is lost and is detected as a loss of heterozygosity (LOH). When this loss involves a tumor suppression gene, carcinogenesis may occur. In prostate cancer, several common sites of allelic loss have been identified including 7q, 8p, 10p, 10q, 13q, 16q, and 18q (1-4). The 8p arm is most frequently lost as 80% of primary and metastatic prostate cancers show this chromosomal variant (10, 11). Most allelic deletions on 8p involve large chromosomal intervals. Working from large collections of clinical specimens, researchers are attempting to define a common overlapping chromosomal region. From this overlapping region, the goal is to then identify particular genes and evaluate their relationship to prostrate cancer.

This approach to allelic loss mapping is difficult if LOH is common and the lesions are complex or non-specific. The approach now used to solve this dilemma is to pursue a random search from available clinical specimens to try to strengthen the correlation of tumor phenotype and chromosome architecture. Another approach to accelerate gene identification in prostrate cancer would be to experimentally test the effect of LOH for particular chromosomal regions on the development of prostate cancer. To date, this approach has not been utilized although it has the potential to greatly speed up the process of prostrate cancer gene identification.

This accelerated approach would involve inactivating areas of the mouse chromosome known to contain sites of allelic loss previously identified to be associated with prostrate cancer. Despite the fact that most human genes have direct homologues in the mouse, the structure of the mouse chromosomes are quite different from the human. Apart from the fact that human chromosomes are acrocentric and the mouse chromosomes are telocentric, the mouse and human genomes show many dissimilarities in the linear arrangements of genes. After years of chromosome mapping by a variety of techniques, a comparative physical and genetic map of the human and mouse chromosomes has emerged (12, 13). It is now possible to draw a direct comparison between subregions of a human and mouse chromosome. For example, human chromosome 8p is distributed in discrete blocks among two mouse chromosomes (mouse ch8 and mouse ch14). These blocks, or syntenic regions, are stable heritable units of genes. Within each chromosomal block, the arrangement of genes is very similar if not identical between mouse and human. Inactivation of these "human chromosome" blocks on the mouse chromosome would allow for physical interval testing to determine their role in prostate cancer. Furthermore by experimentally inducing LOH in the mouse, these syntenic blocks could provide a experimental directed approach to test how a particular region of a human chromosome might operate in the pathogenesis of prostate cancer.

Lessons from X-chromosome inactivation: developmental LOH.

A new technology is required to direct the functional inactivation of a chromosome and create experimental LOH in transgenic mice. We propose to create this technology based upon our basic pioneering work to define the process of X-chromosome inactivation. X-chromosome inactivation is the only known example in mammals of a developmentally regulated functional loss of heterozygosity. It is an example of an epigenetic developmental program that begins anew in the development of every female and represents a unique aspect of an individuals characteristics. X-inactivation is a particular type of epigenetic program operating in female mammals for the purpose of gene dosage compensation between the heterogametic sexes. (14, 15). X-inactivation allows a female embryo to functionally appear as monosomic for the X-chromosome despite the presence of two X-chromosomes. If this process did not occur it would be catastrophic to the developing female embryo with twice the number of X-linked genes as the male.

Two facts are established regarding the mechanism of X-inactivation: (1) a gene which encodes a nontranslateable RNA called *Xist* is necessary for X inactivation (16, 17). Early in development, prior to X-inactivation both male and female cells exhibit a low level of *Xist* expression (18, 19). Subsequent to implantation in female cells one X-chromosome is chosen and exhibits a significant induction of *Xist* expression (18, 19). Soon after *Xist* induction, genes in cis to the actively transcribed *Xist* are repressed for the lifetime of the cell (19-21). If the structural portion of the *Xist* gene is interrupted by homologous recombination, the X-chromosome containing this interrupted allele is incapable of undergoing X-inactivation (16, 17). This demonstrates the necessity of *Xist* for X-inactivation.

A region on the X-chromosome not much larger than the *Xist* gene is sufficient to direct the choice of which X-chromosome undergoes inactivation (19, 21). This DNA interval was first cloned in the form of a yeast artificial chromosome (YAC) by our laboratory and introduced into male embryonic stem cell lines derived (ES cells) (19). This 450 kb YAC was sufficient to be counted as an X-chromosome and direct inactivation on any chromosome in which it was integrated (19). Similarly a 40 kb cosmid was shown to sufficient to cause autosomal inactivation when the cosmid was autosomally integrated in cis in male ES cells (21).

Based upon the observation that a YAC or cosmid spanning *Xist* is necessary and sufficient for X-chromosome inactivation, a vector harboring the *Xist* gene under conditional control could operate to inactivate any chromosome in which it was integrated. To this end we have created a full length cDNA of the murine *Xist* and used it to create a vector in which the cDNA is under control of the tetracycline inducible operator system (22). This vector once integrated into the chromosome of choice would inactivate this chromosome once the *Xist* gene was to be activated. The activation of the *Xist* gene would be controlled by the exogenous administration of tetracycline at any point in development.

The common mouse has been used as a model system for experimental prostate cancer research. Despite the fact that prostate cancer is rarely observed among rodents several approaches to experimental modeling have been developed in the mouse. Three approaches have been described including 1) androgenic hormone stimulation with carcinogen exposure, 2) retroviral transduction and organ reconstitution and 3) transgenic targeting (23). In this proposal we will only address the transgenic approach to prostate cancer modeling. There have been essentially two transgenic models described which show prostate changes characteristic of human disease. These two models involve two different dominant oncogene/protein and two different promoters. The first system involves the use of the MMTV promoter and the Int-2 oncogene. The MMTV promoter is a glucocorticoid responsive viral promoter with favored expression in the mammary tissue of the lactating female. However, it has been shown that male transgenic mouse lines expressing the Int-2 gene under MMTV control results in dramatic epithelial hyperplasia of the prostate (24). The TRAMP model (transgenic adenocarcinoma mouse prostate) has also been described (25, 26). The TRAMP system involves the rat probasin promoter directing expression of the SV40 large T gene in a prostate specific manner. Several reports indicate that this model system recapitulates the aggressive course of human prostatic cancer (25-28). Prostatic intraepithilial neoplasia is observed in male mice of 8 - 12 weeks age. These lesions appear to progress to adenocarcinoma by 30 weeks and finally to distant metastases (25-28).

Preliminary Data

At the time of our Phase I grant submission we had finished the construction of what we believed to be a full length inducible cDNA version of the gene *Xist*. Our construction was guided by the published structures for the *Xist* genomic locus and RNA (34,35). During the final quality control steps, prior to introduction of our construct into ES cells and mice we started an exhastive confirmation process to demonstrate not only that our Xist clone was identical to the published *Xist* structure, but that our clone was identical to the sequences found in the mouse germline. Much to our surprise (and dismay) despite the absolute identity of our clone the published structure, our clone contained discrepancies relative to the mouse genome. We struggled to discover the basis of these differences, and revealed that the published structure for *Xist* was in error and in need of revision. We discovered new structural data for the murine *Xist* gene. These data were published (36), and this paper demonstrates that the murine *Xist* transcript is at least 17.8 kb not 14.7 kb as previously reported. The new structure of the murine *Xist* gene described herein has seven exons, not six. Exon VII encodes an additional 3.1 kb of information at the 3'-end. Exon VII contains seven possible sites for polyadenylation, four of these sites are located in the newly discovered 3'-end. Consequently it is possible that several distinct transcripts may be produced through differential polyadenylation of a primary transcript. Alternative use of polyadenylation signals could result in size changes for Exon VII. Two major species of *Xist* are detectable by Northern analysis, consistent with differential polyadenylation.

Analyzing the human *XIST* structure has resulted in a strong structural correlation between the two organisms (37). Comparison of sequences from the genomic interval downstream to the 3' end of the human *XIST* gene against the human EST database brought to light a number of human EST sequences which are mapped to the region. Furthermore, PCR-amplification of human cDNA libraries and RNA-Fluorescence *In Situ* Hybridization (RNA-FISH) demonstrate that the human *XIST* gene has additional 2.8 kb downstream sequences which have not been documented as a part of the gene. These data show that the full length *XIST* cDNA is in fact 19.3 kb, not 16.5 kb as previously reported. The newly defined region contains an intron that may be alternatively spliced and seven polyadenylation signal sequences. Sequences in the newly defined region show overall sequence similarity with the 3' terminal region of mouse *Xist* and three subregions exhibit considerably high sequence conservation. Interestingly, the new intron spans the first two subregions that are absent in one of the two isoforms of mouse *Xist*. Taken together, we revise the structure of human *XIST* cDNA and compare cDNA structures between human and mouse *XIST*/*Xist*.

Finally, another paper has just been submitted documenting the structural explanation for the two RNA isoforms of murine *Xist* and the most reasonable mechanism for their production (38). To further define the molecular structures of the two *Xist* RNA isoforms, we performed northern blot analyses and RNAse protection assay (RPA). Consistent with previous data, our northern blot analyses show that majority of the two transcripts are directed by P2 promoter. Additionally, the northern probe spanning 853 base pairs sequence 3' of *Xist* gave only one band indicating the two isoforms are different at their 3' termini. Probes for the RPA spanned either originally defined 3' terminus or two of the putative polyadenylation signals at the 3' termini. Results of the RPA experiments clearly show that *Xist* does not end at the previously proposed site, and the two isoforms are different in their sizes which we called short (*S*) and long (*L*) forms. The *S* form ends at 17030 nucleotides from the +1 transcription start site while the *L* form ends at 17873 nucleotides of the *Xist* cDNA. Therefore the *S* form is 843 nucleotides shorter than the *L* form. The following lines of evidences suggest that the difference in length at the 3' termini of the two *Xist* isoforms is due to differential polyadenylation, not splicing: 1) Only one band was detectable with the northern probes (pWS855, 859 and 860) spanning 3' of *Xist*. 2) RPA with P2 probe showed 3' termini of both *S* and *L* forms, and there are putative polyadenylation signals and hairpin structures close to these ends. 3) Analyses of splice site prediction program did not show any evidence of splicing in the sequence of *L* form. The extra sequence of the *L* form shares significant sequence similarity with our revision for the structure of the 3' region of human *XIST*. This suggests that mouse *Xist* depends on differential polyadenylation to generate the two isoforms while human *XIST* may depend on alternative splicing in addition to differential polyadenylation. The newly revised structure of *Xist* isoforms may play essential roles in the stability of *Xist* and the process of X inactivation.

Clearly, the schedule for our prostate project was derailed by the important findings of inaccuracies in the *Xist* structure. Instead of being able to start our transgenic experiments immediately we have spent the last year defining the actual structure of the *Xist* gene and RNA. In addition to defining the true structure for mouse and Human *Xist*/*XIST* it was necessary to rebuild our cDNA constructs. We now report that full length cDNAs for mouse *Xist* (17.8 kb) have been made.

In addition, using the revised *Xist* cDNA three types of expression constructs have also been made. First a vector that expresses *Xist* in a constituitive manner. Second,

two types of inducible *Xist* constructs have been made, 1) a tetracycline regulated form, and 2) an interferon inducible form.

All of three of these *Xist* expressing constructs have been introduced into somatic and ES cells by random transfection for the purpose of expression testing. The somatic cells used for these experiments are NIH 3T3 cells. These immortalized cells have been successfully transfected with the constructs. In each case the *Xist* constructs expressed RNA which we could detect both by Northern and RNA-FISH. The RNA-FISH results were quite exciting as the ectopically derived *Xist* was observed to "coat" or localize on the transgenic chromosome.

A number of different ES cell lines which inducibly express ectopic *Xist* have been produced. These cell lines were characterized to determine the chromosome into which the transgene had integrated. Our current results show random integrations into mouse 4, 5, 8, a number of additional ES cell lines have yet to be characterized. The integration into distal chromosome 8 is especially exciting as this chromosome is directly relevant to our proposed prostate cancer model of LOH.

Experiments to functionally characterize the transfected constructs have been undertaken. Each of the ES cell lines with *Xist* integrations, into either chromosome 4, 5, 8, have characterized by *Xist* localization, and cis-inactivation of gene expression. For chromosome 4 gene specific assay for *c-jun*, *Tlr4*, and *CDC42* were evaluated by RNA FISH. For chromosome 5 gene specific assay for beta-actin, ketokinase, and *CENP-A* were evaluated by RNA FISH. For chromosome 8 gene specific assay for *EIF-4E* and *Aprt* were evaluated by RNA FISH. In the transfected ES cell cultures, when *Xist* is expressed in an inducible manner it localizes to the transgenic chromosome and result in silencing of the genes in cis to the construct.

7) Key Research Accomplishements

Redefinition of murine and human Xist/XIST gene structure
Redefinition of murine and human Xist/XIST RNA structure
Construction of 2 inducible versions of the murine Xist gene.
Transfection of these constructs into mouse somatic and ES cells.
Conditional expression of the inducible version of murine Xist in ES cells.
Demonstrations that Xist cDNA alone will accomplish cis-silencing.
Targeting of muine Chromsome 8 with conditional Xist construct.
Demonstrated that there is only one start site for the Xist RNA.
Demonstrated that there are two different Xist RNA isoforms.
Showed that the Xist RNA L-isoform is the predominant isoform in the preimplantation embryo.
Showed that the Xist RNA S-isoform is polyadenylated
Demonstrated that the Xist RNA L-isoform in not polyadenylated
Showed that there is a stem cell specific Xist RNA L-isoform.
Demonstrated that there are at least five different Xist RNA L-isoforms.
Showed that the Xist RNA L-isoforms found in the stem cell are not found in the somatic cells.

8)Reportable Outcomes

Publications

1. Hong, Y-K, S.D.Ontiveros, C.Chen, W.M. Strauss. A New Structure for the Murine *Xist* Gene and its Relationship to Chromosome Choice/Counting during X-chromosome Inactivation. *Proc. Natl. Acad, Sci. U.S.A.* **96**(12): 6829-6834(1999).

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9) Conclusions

The scientific conclusions of this report are very optimistic. We have redefined the structure for mouse and human Xist/XIST gene and transcript. This transcript causes cis-inactivation of the chromosome from which it is expressed. Thus as we continue to construct the mouse strains harboring the Xist cDNA and the tetracycline transactivator under probasin promoter control we have confidence that the expression of Xist will cause the desired result.

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11)Appendicies

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